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Review

Improving plant-resistance to insect-pests and pathogens: The new opportunities through targeted genome editing

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ABSTRACT

The advantages of high input agriculture are fading away due to degenerating soil health and adverse effects of climate change. Safeguarding crop yields in the changing environment and dynamics of pest and pathogens, has posed new challenges to global agriculture. Thus, integration of new technologies in crop improvement has been imperative for achieving the breeding objectives in faster ways. Recently, enormous potential of genome editing through engineered nucleases has been demonstrated in plants. Continuous refinements of the genome editing tools have increased depth and breadth of their applications. So far, genome editing has been demonstrated in more than fifty plant species. These include model species like Arabidopsis, as well as important crops like rice, wheat, maize etc. Particularly, CRISPR/Cas9 based two component genome editing system has been facile with wider applicability. Potential of genome editing has unfurled enormous possibilities for engineering diverse agronomic traits including durable resistance against insect-pests and pathogens. Novel propositions of developing insect and pathogen resistant crops by genome editing include altering the effector-target interaction, knocking out of host-susceptibility genes, engineering synthetic immune receptor eliciting broad spectrum resistance, uncoupling of antagonistic action of defense hormones etc. Alternatively, modification of insect genomes has been used either to create gene drive or to counteract resistance to various insecticides. The distinct advantage of genome editing system is that it can knock out specific target region in the genome without leaving the unwanted vector backbone. In this article, we have reviewed the novel opportunities offered by the genome editing technologies for developing insect and pathogen resistant crop-types, their future prospects and anticipated challenges.

1. Introduction

In this Anthropocene human being is the steward of global sustainability. At one point of time input intensive agriculture was invented and essential to feed the burgeoning population for the last several decades. Now agricultural productivity is facing the challenge of sustainability in the backdrop of deteriorating soil health and elusive climate change. Second generation challenges of agricultural productivity are confounded with the unpredictable impact of climate change. Thus, sustainability of the productivity has assumed more importance and priority bracketing all the breeding endeavours towards productivity enhancement. Apart from numerous kinds and magnitude of abiotic stresses, biotic stresses like damage due to insect-pests and pathogens constitute major threats to crop productivity and never lost its relevance in global agriculture. Annual loss in agricultural productivity due to damage by insect-pests and diseases play a detrimental role in accomplishing the goal of eradicating poverty and hunger. Breeding insect and disease resistant cultivars is the most environmentally benign and sustainable method of crops protection. However, in many instances lack of resistance source within the accessible genepool limits the scope of breeding resistant cultivars [1,2]. In the absence of genetic resistance, crop protection solely relies on application of toxic agrochemicals which are cost-intensive as well as hazardous to the eco-system. Taking into account the concern of environmental pollution and global climate change it has been impelling necessity to develop genetically resilient plant types [3]. Phenomenal advancement in modern biology in terms of tools and techniques provide the right opportunity of translating biological concepts into novel avenues of developing genetic resistance against insect-pests and pathogen. For example, the universally occurring cellular phenomenon of

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RNA interference (RNAi) has been utilized in devising gene-silencing mediated resistance against insect-pests and pathogens [4,5]. Development of RNAi technology added new possibilities of trait improvement by engineering 'loss- of- function' in targeted manner beyond the limited scope of gene introgression based breeding. However, a major limitation of RNAi technology has been the lack of complete silencing of the target loci. Recently, the expanding arrays of genome editing strategies as a tool for site specific gene-editing presented enormous possibilities of impacting important agronomic traits including resistance to biotic stresses [6,7]. Here we have reviewed the novel opportunities that the distinct advantages of genome editing have offered in developing genetic resistance against insect-pests and pathogens.

2. Genome editing techniques: A decade of refinement, structural improvement and identifying novel variants

Targeted genome editing involves precise manipulation of specific genomic sequences by exploiting different nucleases. There are four major classes of sequence specific nucleases (SSNs): meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced palindromic repeats-associated 9 (CRISPR/Cas9) [8]. Editing strategies began with ZFNs followed by TALENs and now CRISPR/Cas, a bacterial adaptive immune system. The latter has been more of a set of related biological mechanisms that have been put together for the purpose of genome editing [9]. In plants most of the genome editing applications has used TALENs or CRISPR-system. In general, these strategies depend on site-specific recognition of DNA sequences, generation of double strand breaks (DSBs) in the gene/genomic region to be edited with the use of sequence specific nucleases (SSNs) and repair of DSBs by endogenous machinery. The endogenous DNA repair mechanisms primarily include non-homologous end-joining (NHEJ) and homology directed repair (HDR). The NHEJ can introduce insertions/deletions (indels) at the break site while HDR is used to replace broken chromosome with user-defined synthetic construct. Nowadays most of the genome editing applications in plants have used TALENs or CRISPR-system.

2.1. CRISPR/Cas9 mediated genome editing

The CRISPR system is an adaptive immune mechanism present in bacteria which protects it from invading bacteriophages and plasmids [10]. DNA fragment from the invading phages and plasmids are incorporated into the host CRISPR locus as spacer between crRNA repeats. Active transcription of this pre-crRNA followed by its pre-processing by Cas9 and host factors leads to development of a mature crRNA which acts as a guide for Cas9 protein. Hybridization of *trans* - acting crRNA (tracrRNA) with crRNA is essential for pre-processing of pre-crRNA. The mature crRNA-Cas9 complex guides the Cas9 to the matching target locus in the invading DNA. The Cas9 nuclease creates site specific cleavage of target sequence upstream of the PAM motif (Protospacer adjacent motif). The Cas9 from *Streptococcus pyogenes* (SpCas9) is the most commonly used Cas9. SpCas9, to perform cleavage essentially require canonical PAM form 5′ NGG 3′ at the 3′ end of the target site. This natural bacterial immune response system has been repurposed to evolve as a facile site-specific genome editing tool. For genome editing applications the crRNA is fused to tracrRNA to function as chimeric single guide RNA (sgRNA). This sgRNA potentially guide the Cas9 protein to the target locus introducing double stranded breaks within the base pairing region. These double stranded breaks are either repaired by non-homologous end-joining (NHEJ) or homology directed repair (HDR). In absence of the template, the breaks are repaired by NHEJ which often leads to heritable mutation altering the encoded trait [11]. Thus, being a simple two component system, it is easily programmable for mutating specific genomic targets. In plants, there are now several examples where customized gRNA- Cas9 complex has been used to target economically important traits [12].

2.2. Engineered variants of Cas9 for efficient and precise genome editing

Precise genome editing requires improvement of CRISPR/Cas systems for better efficiency, increased specificity and reduced rate of off-targeting. Thus, extensive experimental work has been carried out to modify Cas9 nuclease and to identify Cas9 orthologs. One of the major limitations in use of CRISPR-Cas9 system is the strict requirement of canonical PAM sequence juxtaposed with the target which limits the range of DNA sequences that can be targeted. Several novel cas9 variants have recently been identified from diverse bacterial sources exhibiting diverse PAM specificities [13]. In addition, researchers have also engineered spCas9 that can recognize broader range of PAM sequences including NG, GAA and GAT [14]. These variants provide flexibility of editing different target sites in the genome which are otherwise inaccessible to archetypal spCas9.

Recently, a new CRISP-Cpf1 system has been characterized from *Francisella novicida* U112 [15]. Like Cas9 protein, it also contains endonuclease domain RuvC; however, lacks the HNH domain. Unlike Cas9 the trans acting RNA is not needed for processing of pre-crRNA. The Cpf1 recognize the canonical PAM form 5′TTTV-3′. Upon cleavage of the target site Cpf1 produces staggered DNA double stranded break which makes it an attractive alternative for specific purposes like genomic insertions and deletions [16]. Notably, in a recent study with human genome it was found that Cpf1 have substantially lower genome-wide off-target activity than spCas9 [17]. In soybean, CRISPR-Cpf1 complex has been recently demonstrated as a suitable system for DNA-free genome editing with no off-target activity [18]. This has a special implication in polyploid plant species where due to high level of genome duplication the chances of off-target mutations are very high.

There are four engineered variants of Cas9. They are, 1. CRISPR Nickase that generates single stranded breaks at the targeted DNA sequence [19], 2. dead Cas9 or nuclease deficient Cas9 (dCas9) which has no DNA cleavage activity [20], 3. High-fidelity CRISPR-Cas9 (SpCas9-HF1) that has exceptional precision [21] and 4. enhanced specificity SpCas9 (eSpCas9) which reduce the off-target effects [22]. Cas9 nickase and nuclease deficient dead Cas9 (dCas9) have been derived by functionally altering spCas9. Cas9 nickase introduces single strand break in the genome. Introduction of double strand break using nickase can be accomplished by using paired nickase guided by sgRNA matching the adjacent target site. This is of particular utility where goal is to minimise the off-target activity. In Arabidopsis, Cas9 paired nickase have been effectively demonstrated to edit the target sequence generating stable heritable progeny with minimum off-target activity [23]. The utility of paired nickase in deleting large fragments up to 1 kb, as demonstrated in case of human genome [24], can assist in removing the unwanted linkage drag in plants which is sometime associated with desirable traits particularly when they have been introgressed from wild relatives. Similarly, another engineered variant of Cas9 is catalytic dysfunction Cas9 (dCas9). In dCas9, the nuclease activity of spCas9 is completely abolished [25]. The dCas9 has much broader application in plants including transcriptional activation and suppression of the genes [26,27], epigenetic editing [28] and in situ hybridisation studies. Another distinguished achievement is fusion of dCas9 with activation-induced cytidine deaminase (AID) that enables site specific mutations at a single base resolution [29,30]. This target AID-mediated mutagenesis (TAM) complex can be utilised in generating large repertoire of variants at desired loci and thus promising its future applications in plant biology. This is of seminal importance in plant biology where the goal is to develop a large number of R-gene variants and thus to cope up with the rapid rate of pathogen evolution.

In addition, novel CRISPR systems have been developed comprising of RNA guided endonucleases from *Prevoltella* and *Francisella*1 (Cpf1) [15], *Campylobacter jejuni* (Cj) [31], *Staphylococcus aureus* (Sa) [19], *Treponema denticola* (Td) [32], *Streptococcus thermophilus* (St) [33], and *Neisseria meningitides* (Nm) [34]. Each of these has different Cas enzyme, PAM site and gRNA backbone.

CRISPR/Cas9 can also cleave single stranded RNA, provided the PAM sequence is present in *trans* as a separate oligonucleotide. Upon recognition of PAM presenting oligonucleotide (PAMmers) by Cas9-gRNA complex, site specific endonucleolytic cleavage of ssRNA is initiated. The PAMmers sequence can be customized according to the target sequence and can theoretically stimulate cleavage of any kind of ssRNA molecule. Interestingly, this RNA recognition property of spCas9 has been further expanded to study the live tracking of RNA molecule in living cells [35]. The creation of Cas9 variants via recombinant DNA technology and exploration of Cas9 orthologues has enabled this technology to position itself at the forefront among other contemporary genome editing strategies.

3. Genome editing in plants: obtaining successful modification

A wider application of genome editing technologies and democratization of CRISPR/Cas9 has undoubtedly taken over the frontier areas of plant research. Although, the initial customization of the sgRNA and Cas9 was confined to its application in mouse and human cell lines, gradually it was picked up by the plant researchers and with subsequent plant specific modification of genome editing components. Nowadays, a simplified version of methodology for genome editing comprises of a single guide RNA (sgRNA) generated by the fusion of two RNA moieties viz. CRISPR-RNA (crRNA) and *trans*-activating CRISPR-RNA (tracrRNA) and an engineered nuclease Cas9. sgRNA binds to the 'protospacer adjacent motif' (PAM) on the targeted sequence and guide Cas9 for specific cleavage at the PAM sequence NGG (N is A, T, G or C). Selection of appropriate target sites and specificity of CRISPR/Cas9 system are the most important concerns for achieving desired genome modifications. At present, there are several successful examples where agronomically important traits have been engineered in crops like rice [36], maize [37] and wheat [38]. Despite the unprecedented success there are several challenges in adapting this technology in agricultural research particularly with the crops that are asexually reproduced and those which are recalcitrant to transformation. Several efforts are underway on the fine tuning of CRISPR/Cas9 based tools for obtaining precise editing of the target locus in plant genome.

3.1. Vector design

Customized vectors have been designed for the specific goals. Mostly, an RNA polymerase III promoter, the promoter of U3 and U6 small nuclear RNA genes is used for driving the expression of sgRNA in CRISPR/cas9 vectors [39]. It is important to mention that choice of promoter is pivotal in attaining high mutation efficiency in the target cells. Generally well characterised U6 promoter of rice like *OsU6a, OsU6b* and *OsU6c* for the monocots and Arabidospsis promoters, *AtU6-1* and *AtU6-29* for the dicot species are preferred for driving the sgRNA expression [40]. Likewise, the delivery of Cas9 into the cell can be achieved either by cloning it downstream to the sgRNA cassette driven by an independent RNA polymerase II promoter or by a separate plasmid harbouring the Cas9 gene with regulatory elements for its expression. Generally, Cas9 is fused with single or dual nuclear localization signal (NLS) to target nuclear DNA [29]. Codon optimization of the native Cas9 protein is also a major determining factor in obtaining optimal editing efficiency in the eukaryotic cell [41]. Many previous studies in plants have used Cas9 codon optimized for human genome editing. However, now there are several examples of Cas9 gene specially codon optimised for efficient cleavage in plants [42,43,44].

3.2. Optimising delivery methods of CRISPR/Cas9 cassette

Efficient CRISPR based editing requires co-delivery and expression of Cas9 protein and its cognate sgRNA in the target cell lines. Conventional plant transformation methods, like particle bombardment and *Agrobacterium tumefaciens* mediated delivery can be adapted for transfer of Cas9 and sgRNA cassettes in callus, mature embryo or other tissues [29]. Preliminary screening for evaluating the editing efficiency for a CRISPR/Cas9 cassette, can be done via protoplast transfection or by Agro-infiltration of tobacco leaves [40,43,45]. The functional cassette(s) identified in transient assay can subsequently be mobilised for the development of stable transgenic plants expressing the Cas9 and sgRNA. It is always desirable to have edited plant free from any footprint of foreign DNA. For that, in the subsequent generations a desired mutant segregated from the sgRNA: Cas9 cassettes can be isolated. Moreover, removal of vector DNA sequences by breeding is not feasible for the crops that reproduce asexually. Interestingly, the desired editing can be achieved by delivering preassembled ribonuclear proteins (RNPs) into the target cells without incorporating sgRNA:Cas9 DNA cassettes [46]. An additional benefit of using RNPs in genome editing is that as this is a DNA free approach, in future it might be exempted from the purview of regulatory legislations for GMOs. Moreover, as protein and its cognate RNA are directly transfected into cell there is no need of codon optimization and specific promoter for driving gene-expression. In spite of alternative delivery methods, lack of established plant transformation protocol for recalcitrant crops and genotype dependence of transformation efficiency are still considered major impediments in realising actual potential of genome editing technology in crop improvement. Interestingly, a recent study conducted in maize demonstrated that overexpression of *Baby boom (*Bbm) and *Wuschel2* (Wus2), genes from maize (*Zea mays*) increases transformation efficiency in non-transformable maize inbred lines, maize, sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*), and indica rice (*Oryza sativa* ssp. indica) [47]. Such novel findings will surely pave the way for future application of genome editing in the crops which are otherwise non-transformable.

Moreover, along with rapid refinements in the genome editing technology, identification of the target genetic points is equally important. It will essentially require advancement in understanding the basic cellular processes and their convergence into trait manifestation. In this perspective, a broader understanding of insect and disease resistance genes as well as their linkage to the complex network of plant immunity, is pivotal in conceptualizing novel possibilities of improving the crop performance.

4. Cellular mechanism of plant defence against insect-pests and pathogens

Being sessile, plants are equipped with a complex network of immune response to fend off most of the insect and pathogen attacks. Initial pathogen attack is sensed by the cell surface receptors called pathogen recognition receptors (PRRs). These PRRs recognize conserved microbial molecules known as pathogen associated molecular patterns (PAMPs) a few of which are well characterized such as bacterial flagellin or fungal chitin [48]. In case of insect invasion such molecules have been termed as herbivore associated molecular patterns (HAMPs) [49]. The PRRs are typically leucine rich repeat kinases and lysine motif kinases [50]. The PAMP/HAMP-PRR interaction elicits the first layer of plant defence called as pathogen triggered immunity (PTI) that restricts the microbial colonization. A successful pathogen however, overcomes PTI to shuttle specific effectors into the plant cell and hijack the cellular machinery for their own subsistence. To counteract, plants have evolved a second layer of defence, called effector triggered immunity (ETI) [50]. ETI is mediated by intracellular resistance receptor (*R*) protein, mostly having NBS-LRR domain, activated against specific effectors [51,52]. The effector triggered activation of R gene often leads to localized cell death or a hypersensitive response (HR) and thus prevents further colonization of the pathogen [53].

In resistant breeding, *R* genes have been extensively utilised and most of the time they are introgressed from sexually compatible closely related species or wild relatives. However, due to emergence of new virulence determinants in pathogen, the durability and longevity of

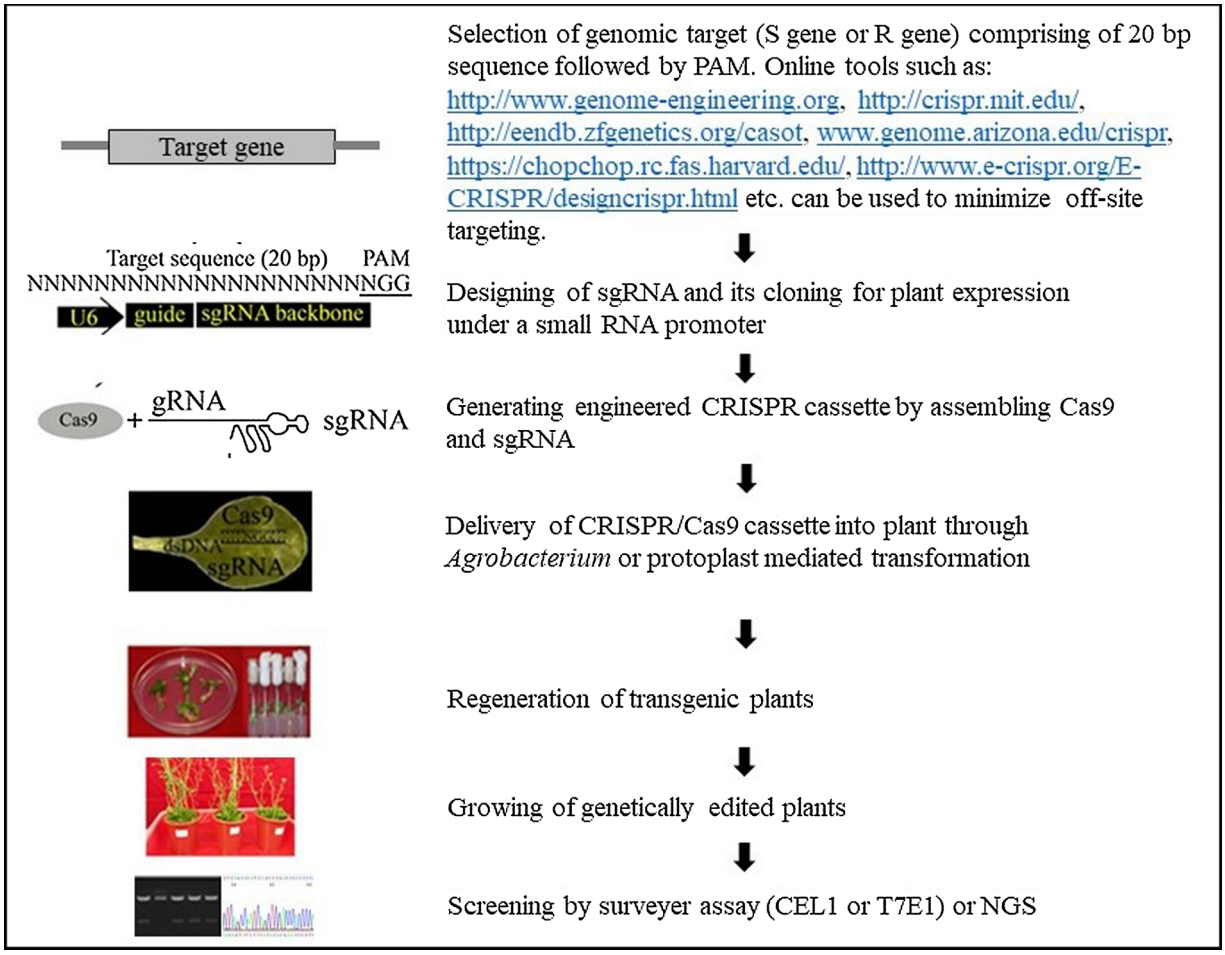
Target gene
Target sequence (20 bp) PAM
NNNNNNNNNNNNNNNNNNNNGG
U6
guide
sgRNA backbone
Cas9
+
gRNA
sgRNA
Cas9
dsDNA
sgRNA
Selection of genomic target (S gene or R gene) comprising of 20 bp sequence followed by PAM. Online tools such as: http://www.genome-engineering.org, http://crispr.mit.edu/, http://eendb.zfgenetics.org/casot, www.genome.arizona.edu/crispr, https://chopchop.rc.fas.harvard.edu/, http://www.e-crispr.org/E-CRISPR/designcrispr.html etc. can be used to minimize off-site targeting.
Designing of sgRNA and its cloning for plant expression under a small RNA promoter
Generating engineered CRISPR cassette by assembling Cas9 and sgRNA
Delivery of CRISPR/Cas9 cassette into plant through Agrobacterium or protoplast mediated transformation
Regeneration of transgenic plants
Growing of genetically edited plants
Screening by surveyer assay (CEL1 or T7E1) or NGS

Fig. 1. Workflow for CRISPR/Cas9 based generation of insect resistant plant.

resistance conferred by the *R* gene is often compromised [54]. Alternatively, accessing genes from distant organisms through transgenic strategy have offered a potential avenue for overcoming many of the bottlenecks associated with breeding resistance [55]. In fact, there are several strategies through which disease and insect resistance can be engineered in crops. For example for disease resistance, expressing R-genes, pathogenesis-related (PR)/ antimicrobial genes, detoxification of pathogen virulence factors, increasing structural barriers, etc. and for insect resistance, expressing insecticidal bacterial genes, antinutritional proteins like protease inhibitors, lectins, host-delivered RNAi and the modification of defence- signalling pathways etc. [54]. However, despite a wider cultivation of insect and virus resistant transgenic plants the bacterial and fungal resistant transgenics have received limited success [56]. Often the failure is associated with, low level of resistance imparted by the transgene or the resistance being generally restricted to a single race or strain [55,57]. In spite of overcoming technical impediment, field testing and release of transgenics engineered for insect, bacterial and fungal resistance critically suffer negative public perception. Such negativity is primarily due to opposition in general for the genetically engineered crops and because of ignorance of the people about scientific principles.

Deployment of genome editing for improving resistance to biotic agents may vary on case-by-case basis. Such approaches may include, a) altering the effector-target interaction, b) knocking out of susceptibility genes, c) modulating the expression of genes involved in maintenance of insect feeding or pathogen progression and d) engineering synthetic *R* gene variants recognizing broad range of insects or pathogens. Traditional mutagenesis approach of knock-down following loss of function mutation is a straightforward approach of enhancing disease resistance in crops, albeit extremely laborious and time consuming. A famous example is mutation induced recessive alleles (*mlo*) of barley *MLO* locus which confers broad spectrum resistance to Powdery mildew [58]. Notably, a more convenient and swift method of conferring resistance to powdery mildew was demonstrated in wheat where *Ta-MLO* homeoalleles were efficiently knocked down using genome editing toolbox [38].

5. Genome editing in improving plant-resistance to insect-pests

Insect-pests adversely damage plant growth and development directly and often indirectly by transmitting pathogenic viruses eventually leading to significant losses in crop yields. The prevalent agrochemical-based control methods are cost intensive and environmentally hazardous. It also negatively affects non-target insects such as pollinators, bio-control agents and encounters gradual inefficacy due to evolution of insecticide resistance. In many instances, the scope of breeding insect resistant crops is limited primarily due to non-availability of well characterized resistance source within the crossable gene pool. In mitigating this bottleneck several efforts aim for accessing genes from wild relatives and uncharacterized accessions of crop plants. However, much success could not be achieved because of poorly understood genetics of the resistant trait in uncharacterized accessions [59]. Alternatively, transgenic approach has been used for the introduction of insect resistance genes from other distant sources into the crops, for example, Bt genes of bacterial origin. However, such transgenic plant types suffered from serious political, ethical and societal resistance due to lack of scientific knowledge. In this situation, the major challenge for present day agriculture is to develop environment

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friendly breeding tool for crops with the potency to resolve two-pronged breeding objectives: to create *de novo* resistance where suitable R-genes are not available; and to control pest dynamics by breaking insecticide resistance, killing or inducing sterility in inflicting insects. In this regard, genome editing platforms has the potential to generate designer plants particularly in situation where a targeted knock out is likely to generate elite and superior traits or to initiate a gene drive for selectively propagating the mutations leading to lethality of female species of the insect population.

5.1. Genome editing targets for improving plant-resistance to insect pests

Genome editing technology has been successfully applied in diverse range of organisms including insects since many years. However, recent advancement in precise application of this technology envisages it replacing random chemical and radiation mutagenesis methods. Genome editing for insect control by spreading a gene drive requires knock out of target sites in the insect genome that may prove to be lethal, impair sex-ratio or fecundity of impeding insects. Alternatively, editing key genes of plant immunity viz. susceptibility genes (S-genes), resistance genes (R-genes) and the genes controlling the interaction between the target and the insect-effector may also be utilized for developing host-immunity to the insect-pests. Till now most of the researches are hovering around optimizing and fine tuning the components of CRISPR/Cas methods in individual crops. Nevertheless, the leads from basic researches on plant-insect interactions offers vast possibilities of developing insect resistance using CRISPR/Cas9 based genome editing.

A typical workflow has been proposed for CRISPR/Cas9 based generation of insect resistant plant in Fig. 1.

There are many examples in which modification of insect genomes has been used for pest control and to counteract resistance to various insecticides with the use of CRISPR/Cas9 genome editing tool (Table 1). Replacement of insecticide resistance alleles with sensitive ones allows further use of otherwise redundant insecticides.

5.2. CRISPR/cas: specificity, efficiency and off-targeting

Stable integration of sgRNA: Cas9 and its constitutive or tissue specific expression might result into editing of off-target sites. Specificity of engineered nucleases largely depends on hybridization of gRNA with target DNA and interaction of Cas9 with the PAM proximal region [74]. However, efficiency of cleavage of both target and non-target largely depends on nuclease activity, availability of target site and binding affinity between guide RNA and target sequence. When an essential gene is targeted, the editing efficiency can be lowered by using promoters (nanos and vasa) expressing Cas9 specifically in germ cells and not somatic cells thereby preventing occurrence of bi-allelic mutations leading to embryonic lethality. Whereas, less important genes may be edited with U6 promoter driven system that offers highest efficiency. Off-targeting comprise of unintended genome modifications that arise through the use of engineered nucleases and is dependent on the number, position and distribution of mismatches in the guide sequence beyond the first 8 to–12 nucleotides of the guide sequence proximal to the PAM. The mismatches in the PAM-distal region are tolerated. However, the tolerance of Cas9 to these mismatches relies on the amount of its expression in the cell. An increase in off-site targeting is reported with high concentrations of the Cas9 enzyme while its low concentrations increase specificity along-with a reduced on-target cleavage activity [75,76]. Ren et al. [77] reported non-tolerance of three mismatches between gRNA and targets in generating DSBs in *Drosophila*. Therefore, a prior bioinformatics analysis is indispensable to minimize off-target effects of genome editing systems. Target sites should be selected carefully avoiding sites of repeated sequences and sites having high homology with other regions of the genome. Several online platforms and resources are available to design gRNAs such as CRISPR MultiTargeter [78], CRISPR-P [79], while flyCRISPR, CRISPR flydesign, DESKGEN are available particularly for model organism, *Drosophila*.

5.3. CRISPR/Cas: Delivery methods and efficiency evaluation

A safe and efficient DNA delivery method is pivotal for the success of genome editing. The delivery method employed may be direct delivery of RNA and Cas9 or indirect by designing new vectors for generation of transgenics. The delivery of sgRNA in insects can be as plasmid DNA, RNA or encoded in the genome. A plasmid construct comprises of three elements viz. a ubiquitous or tissue/development specific promoter, a Cas9 gene and /or gRNA sequence and a terminator. Also, a nuclear localization signal (NLS) is essential for the expression of Cas9. A large number of plasmid constructs are available at https://www.addgene.org/. The Cas9 mRNA and gRNA can also be prepared by in vitro transcription under the control of T7 or SP6 promoter.

Primarily, microinjection is applied to inject DNA, RNA or protein into early-stage embryos of insects [80] while transfection is mostly used with insect cells [81]. Han et al. [82] developed microfluidic membrane deformation method to deliver Cas9-gRNA in hard-to-transfect cells. Electroporation [83] and oviductal nucleic acids delivery system [84] has been established as an efficient delivery method in mice. However, their feasibility has to be explored in case of insects. In addition, CRISPR/Cas9 technology introduces an exhaustive list of viral [85] and non-viral vectors [86,87] that deliver RNA and Cas9 into cells and tissues thereby generating transgenic strains/lines. Development of transgenic *Drosophila* strain expressing both Cas9 and gRNA in the germline has been demonstrated [88].

The efficiency of mutagenesis can be evaluated by endonuclease

Table 1
Insects engineered for pest management using CRISPR/Cas9 system.

S.No.	Name of the insects	Target gene	Editing	Transformation method	Reference
1	*Plutella xylostella*	*Abdominal*-A	Indels	Embryo microinjection	[60]
2	*Drosophila melanogaster*	*Chitin synthase* 1	Substitution	Embryo injection	[61]
3	*Drosophila melanogaster*	*Nicotinic acetylcholine receptor* α6	Substitution	Embryo injection	[62]
4	*Spodoptera exigua*	*Ryanodine receptor*	Substitution	Embryo microinjection	[63]
5	*Aedes aegypti*	*Male-determining factor (M factor, Nix)*	Knock-out	Embryo injection	[64]
6	*Culex quinquefasciatus*	*cytochrome P450 gene, CYP9M10*	Knock-out	Embryo injection	[65]
7	*Anopheles gambiae*	*rDNA*	Knock-out	Embryo injection	[66]
8	*Anopheles gambiae*	*AGAP005958, AGAP011377, AGAP007280*	Knock-out, Knock-in	Embryo microinjection	[67]
9	*Anopheles stephensi*	*Kynurenine hydroxylase*	Knock-in	Embryo microinjection	[68]
10	*Locusta migratoria*	*Odorant receptor co-receptor (Orco)gene*	Knock-out	Microinjection	[69]
11	*Drosophila suzukii*	*White (w) and Sex lethal(Sxl) genes*	Knock-out	Embryo injection	[70]
12	*Tribolium castaneum*	*E-cadherin gene, EGFP*	Knock-out, Knock-in	Embryo injection	[71]
13	*Spodoptera litura*	*Abdominal-A (Slabd-A) gene*	Knock-out	Embryo injection	[72]
14	*Spodoptera littoralis*	*Olfactory receptor co-receptor (Orco) gene*	Knock-out	Egg injection	[73]

cleavage at mismatch sites resulting in shorter fragments visualized through gel electrophoresis. The endonucleases include CEL1 nuclease family (CEL1 assay or Surveyor assay) or T7 endonuclease1 (T7E1 assay). High resolution melting analysis (HRMA) produces different melting curve for fragment of interest in presence of mismatches in comparison to wild-type leading to detection of mutations in less time with higher sensitivity [89]. Furthermore, sequencing is a method of choice for precise mutation identification while observation of mutant phenotype is the ultimate way to evaluate efficiency [90].

5.4. CRISPR/Cas: Applications and implications in plant-insect resistance

Genome editing system is being used to facilitate several different types of genome modifications including incorporation of point mutations similar to single nucleotide polymorphisms (SNPs), substitution of individual gene segments, insertion of new foreign genes in specific locations (Knock In; KI), deletion of large chromosome regions and knock out (KO) gene functions. Introduction of genomic modifications has also enabled the scientists to investigate the role of individual genes in the functioning of cells and/or organisms. With the use of CRISPR/Cas9 system, crops are being edited for improved/ high nutritional value and production of commercial products including biofuels [91], conferring abiotic stress resistance [92], early yield [93], extended shelf life [94], resistance to biotic stresses [38,95], herbicide resistance [96], synthesis of human proteins, incorporation of complex multigenic traits and pyramiding of genes. Additionally, CRISPR/Cas9 system is also utilized for a variety of other applications including gene expression regulation in specific tissue, in specific developmental stage or in different environmental conditions by activating or repressing the activity of promoters that control their expression, visualization of chromosome architectural changes during development using fluorescent labelled dCas9, epigenome editing by selection of proteins involved in histone modification and DNA methylation, identification of regulatory proteins bound to specific DNA sequences controlling the expression of genes, inhibition or stimulation of the function of target gene, investigation of inter-genic interaction by targeting several genes simultaneously.

In insect studies, CRISPR/Cas technology is being widely applied to model organism, *Drosophila* for genome-wide functional screens. Other insect species are also being targeted for establishment of control strategy for pests. Recently, gene drive; a novel approach to rapidly disperse edited genes in the entire population of target insect species has been developed. CRISPR/Cas based gene drives can cause sterility or lethality upon gene disruption in targeted populations. These gene drives eventually cause population crash or even extinction owing to the excessive load of recessive lethal mutations. Likewise, a gene drive can also distort the sex ratio towards males by specifically destroying X-chromosome during spermatogenesis. This ensures occurrence of Y-chromosome in most of the viable sperms thereby producing more male progeny with progressive reduction in the number of females. Thus, genetic control of insect pest populations through the release of insect strains carrying deleterious traits (biased sex ratio, lethality, infertility, insecticide sensitivity) and introgression of virus resistance genes in insect vectors preventing transmission of viral diseases are the promising strategies for control of insect-pests and their vectored diseases. Over these years, there is a dramatic expansion in the applications of CRISPR/Cas9 system and an assessment of the challenges posed by this technology and its future prospects will help in exploring its suitability for sustainable pest management.

6. Genome editing for engineering disease resistance in plants

Plant pathogens poses a serious challenge to global agriculture by significantly reducing the annual agricultural productivity. Breeding varieties with resistance that is not only effective against diverse class of pathogen but also stable and durable is challenging. The knowledge of disease resistance genes and the complex network of plant immunity, can be siphoned into the ambit of modern biotechnological tools like genetic engineering and genome editing for improving disease resistance in crops. To propose the new possibilities of genome editing in this context, we first describe what has been attained by decades of resistance breeding programs and what is yet to accomplish and how CRISPR/Cas9 meditated genome editing technology can complement.

6.1. Breeding for disease resistance in the pre-editing era

Classical plant breeding has proven adept in improvements of crop cultivars. There are several examples where remarkable efforts by breeders have resulted into the development and release of disease resistance crop varieties [97]. The advent of DNA markers has further assisted breeders in shortening the breeding cycle of varietal development program consequently enhancing the efficiency and precision of conventional plant breeding. However, despite phenomenal success of classical plant breeding it becomes altogether infective in the improvement of disease resistance traits in cases where natural allelic variations are lacking amongst the crossable species. Mutation breeding has been successful to some extent for creating novel genetic variations. However, being a random process, the evaluation and identification of desirable mutants is extremely laborious and time-consuming process. Moreover, once the desirable mutants are identified subsequent breeding steps are required to achieve homozygosity and to remove undesirable mutations [98]. Additionally, it has been frequently noticed that the durability and longevity of resistance imparted in the crop varieties via the above-mentioned means is often short lived, largely due to the evolution of novel pathogenic determinants in the pathogen. This compels plant breeders to breed new crop varieties, on a regular basis, responding adequately to the evolution of virulent strains [99]. Development of genetically modified crops (GM) through recombinant DNA technology provided a promising complementary avenue to traditional breeding as it envisages transfer of beneficial gene(s) across the genus. Globally, during 1992 and 2015 there has been a 100-fold increase in hectarage of GM crops [100]. In case of disease resistance, GM crops resistant to viral diseases are commercialized so far [101]. Unfortunately, the GM crops have ensued a debate on their cultivation as they are developed using foreign DNA and strong legislations have been framed in the name of regulatory requirements for environmental release in several countries. Therefore, it became more challenging to utilize GM technology striking a fine balance between scientific need for ensuring food security and socio-political issues. The toolbox of genome editing technologies offers new possibilities in addressing the food security challenge and simultaneously the core issues of the GM legislations.

6.2. Prospects of engineering disease resistance in plants in the era of genome editing

Genome editing systems can introduce stably inherited point modifications at precise locations in plant genome. Site-specific nucleases are capable of targeted modification including elimination of genes, altering regulatory sequences controlling the gene expression, introduction of desired genes at defined loci and stacking multiple genes into crops with low segregation risk and no undesirable footprints (as in the case of loxP or attB sequences). CRISPR/Cas technology has been used to generate marker-free (without antibiotic resistance genes) [102] and excess genetic material (remnants of plasmids)-free genetically modified crops [103]. These aims are almost unlikely to achieve through traditional breeding methods or even transgenesis. In transgenics, the transgene is subject to position effects where the transgene-expression is influenced by the regulatory sequences and chromatin structure neighbouring the integration site. Further, homology between the transgene and the endogenous gene and other features of transgenic locus viz. transgene copy number, presence of inverted repeats and

truncated sequences substantially increase the probability of silencing the transgenic locus. Thus, the possibility of developing an 'universal recipient line' with a 'specific integration locus' ensuring strong expression of the transgene without undesired effects to plant health will possibly accelerate the production of potent genetically modified lines.

As contemporary technique RNA interference (RNAi) has been universally used for knock-down of gene expression at post-transcriptional level. However, this technology suffered from a few bottlenecks viz. incomplete knockdowns of transcripts, competition with endogenous RNA-mediated gene regulation and unpredictable off-targeting [104]. In contrast, type III-B CRISPR/Cas system from *Pyrococcus furiosus* is a unique and more specific RNA silencing system. It involves homology-dependent degradation of complementary RNA without relying on host factors such as dicer or RISC components and also consequently minimizing the silencing of off-target genes.

In this context, CRISPR interference (CRISPRi) is a strategy reported to repress multiple target genes simultaneously by blocking transcriptional elongation, RNA polymerase or transcription factor binding through the use of dCas9-guide RNA complex. CRISPRi-directed transcriptional repression is highly specific and its effects are reversible. RNAi silencing when applied to silence genes of the RNAi pathway itself (Dicer, Argonaute) leads to difficult interpretation of results due to several indirect and direct effects. But lack of CRISPR/Cas system in eukaryotes which negates any chance of its competition with the endogenous pathways indicates superiority of the CRISPRi over RNAi. Additionally, the ability of CRISPR/Cas system to differentially target either DNA or RNA and to up- or down-regulate gene expression at the transcriptional or post-transcriptional level highlights more versatility of this technique over RNAi which is mostly restricted to attenuating gene expression. In CRISPR/Cas9 system, for knocking out a target gene at its genomic loci a single guide RNA is capable of introducing DSBs; whereas, a pair of proteins is required for inducing a DSB in case of TALENs and ZFNs. Also, owing to large size TALENs and ZFNs are less suited to multiplex gene editing. However, in CRISPR/Cas 'multiplex gene editing' involving simultaneous editing of more than one target at the same time is possible with the delivery of multiple small sized sgRNAs with Cas9 into the cell. Targeted gene insertion as well through homologous recombination (HR) has been demonstrated in several crop plants including tobacco, maize and rice [43,96,42]. Features like simplicity, specificity, efficiency, time saving, eco-friendly, minimal off-target effects, amenability to multiplexing and wide applications differentiate genome editing systems from other contemporary technologies used for genome modification.

The single stranded nucleases (SSNs) provide numerous opportunities of engineering disease resistance in plants. Initially, editing of disease susceptibility factors for reduced pathogenesis was adopted as a straight forward approach for imparting broad-spectrum disease resistance [105]. However, with the evolution of CRISPR/Cas9 systems the repertoire of potential targets in the plant genome has also expanded. The possible targets and their successful editing are discussed in following sections.

6.2.1. Targeting susceptibility genes for disease resistance

Pathogens require host susceptibility genes (S) for successful infection, establishment and proliferation [106]. On the basis of various steps involved in pathogen attack to disease establishment the S genes could be divided into three sub-categories: (i) genes involved in assisting penetration of pathogen into the host; (ii) genes involved in post penetration providing support for pathogen subsistence; and (iii) genes which are negative regulators of immune signalling [107]. Disrupting the susceptibility genes therefore might limit the ability of pathogen to cause the disease. When mutated, S gene meditated disease resistance could be pathogen specific if the mutation results into impairment of the penetration, pre-penetration or post-penetration requirements for a specific class of pathogen [105]. Alternatively, the mutation in S gene could manifest into broad spectrum resistance if the targeted gene evoke prolonged or constitutive defence response [108].

The disruption of S gene has been exploited economically for the development of disease resistance in plants. A prime example being mutation induced recessive alleles of barley *Mildew Locus* (*Mlo)* locus conferring broad spectrum resistance to powdery mildew fungi in the field [58]. Additionally, the homozygous mutations in *Mlo* locus of Arabidopsis [109], tomato [110] and pea [111] have also been shown to evoke immune response to PM fungi. *Mlo* encodes a membrane protein with seven transmembrane domains largely conserved across the plant kingdom [112]. The efficient use of TALEN and CRISPR-Cas9 to introduce mutations in the three homeoalleles of the susceptibility gene *Mlo* in wheat resulted into broad spectrum resistance to PM fungi *Blumeria graminis* f. sp. *tritici* (*Bgt*) [38]. Similar targeting of *Mlo* locus, by CRISPR – Cas9 has also been demonstrated in grape vine, apple and tomato [113,114]. Plant S genes have also been targeted to trigger immunity against bacterial pathogens. Cas9/sgRNA induced frameshift deletion in the DMR6 gene displayed resistance against different pathogens including *Pseudomonas syringae*, *Phytophthora capsici* and *Xanthomonas* spp, with no significant effect on plant growth and development [115].

Targeting of disease susceptibility factors has also been successful in imparting resistance against viral diseases of crops. eIF4e translation initiation are essential plant factors allowing most single-stranded positive sense RNA viruses to infect plants [116]. Consequently, they have emerged as a potential target for genome editing leading to virus resistant plants. In two separate studies it has been demonstrated that disrupting eIF4Es with the CRISPR– Cas9 system can trigger ipomo virus as well as potyvirus resistance in Arabidopsis [117] and cucumber [118]. Likewise, rice tungro spherical virus (RTSV) resistance in rice is also a manifestation of recessive mutant allele in the translation initiation factor 4 gamma (eIF4G) [119]. In a recent study, Cas9/gRNA mediated editing of eIF4G resulted into a novel allele of eIF4G that led to conferring resistance against RTSV [36]. Moreover, the genome-edited plants were free from off-target mutations.

Apart from targeting the coding region, the gene editing of effector binding element has also been identified as a potential target for engineering disease resistance. Bacterial leaf blight is a widespread vascular rice disease caused by *Xanthomonas oryzae* pv. *Oryzae*. *OsSweet14* gene is a major susceptibility factor during rice -Xoo interactions. Pathogen virulence factor, called as TAL (Transcription activation like) upon introduction into the host nucleus binds to effector binding element present in the promoter region of *OsSweet14* gene activating its transcription eventually favouring the proliferation of pathogen [120]. Disrupting the EBE in the promoter sequence by TALEN confer resistance against bacterial leaf blight [121]. Similarly, use of CRISPR/Cas9 displayed successful disruption of *OsSweet 13* and thus preventing the interaction of TAL effector gene pthXo2 leading to improved resistance against BLB in rice [122]. In another recent study, the effector binding element of citrus canker susceptibility gene *CsLOB1* (Citrus *sinensis* lateral organ boundaries) was edited using Cas9/sgRNA with an objective of developing canker resistant citrus varieties [123]. Several other disease susceptibility genes have been identified in variety of crops [108]. A representative list of plant disease susceptibility factors engineered through genome editing is provided in Table 2. However, pertaining to the fitness penalty associated with S gene mutation it becomes important to critically evaluate the desirable mutants before their release for commercial cultivation.

6.2.2. Synthetic immune receptor: Engineering immune receptor for broad specificities

Conventional breeding for disease resistance relies on identification of R gene and their simultaneous introgression in the respective cultivated varieties. However, majority of the times the resistance imparted by the R gene is strain specific and is overcome by the pathogen with the evolution of new strain(s). The main challenge to the breeders is to develop new varieties with novel resistance determinants rapidly

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Table 2
A list of plant host factors edited using CRISPR/Cas9 for engineering disease resistance in plants.

Plant Species and its host factor	Gene Function	Engineered Resistance for pathogen	Mode of Editing	Reference
Triticum aestivum; MLO-A1	Susceptibility gene involved in pathogenesis of Powdery mildew pathogen	Powdery mildew (*Blumeria graminis* f. sp. *tritici*)	CRISPR/Cas9	[38]
Solanum lycopersicum; MLO -1	Susceptibility gene involved in pathogenesis of Powdery mildew pathogen	Powdery mildew (*Oidium neolycopersici*)	CRISPR/Cas9	[114]
Vitis Vinefera; MLO-7	Susceptibility gene involved in pathogenesis of Powdery mildew pathogen	Powdery mildew	CRISPR/Cas9	[113]
Solanum lycopersicum; DMR6	It belongs to the superfamily of 2-oxoglutarate Fe(II) dependent oxygenases and is specifically up-regulated during pathogen infection	*Pseudomonas syringae*, *Phytophthora capsici* and *Xanthomonas* spp,		[115]
Cucumis sativus L.; eIF4E (eukaryotic translation initiation factor 4E)	It is a host factor facilitating the transcription of uncapped viruses having viral protein VPg facilitating virus infectivity	Cucumber vein yellowing virus (Ipomovirus), Zucchini yellow mosaic virus and Papaya ring spot mosaic virus-W	CRISPR/Cas9	[118]
Oryza sativa var. *indica* cv. IR64; eIF4G, (eukaryotic translation initiation factor 4 gamma gene	Plays a role in the assembly of basal translational initiation factors at the 5′-end of mRNA as well as in the cap-independent translation of viral RNA genomes	Rice tungro spherical virus (RTSV)	CRISPR/Cas9	[36]
Arabidopsis thaliana; eIF(iso)4E	Potyviral VPg (Viral protein genome linked) bind to eIF(iso)4E and aids translations of viral genes	Potyvirus	CRISPR/Cas9	[117]
Oryza sativa; SWEET14	SWEET14 is a sucrose-efflux transporter gene whose activation by bacterial effectors is needed to satisfy the pathogen nutrient requirements	*Xanthomonas oryzae* pv. oryzae (Xoo)	TALEN- based disruption of the effector binding elements in the promoter region of OsSWEET14	[121]
Citrus sinensis; CsLOB1 (*Citrus sinensis* Lateral Organ Boundaries)	CsLOB1gene is a susceptibility gene induced by the virulence factor of Xanthomonas citri	Citrus canker caused by *Xanthomonas citri subspecies citri (Xcc)*	CRISPR/Cas9 based disruption of effector binding elements in the promoter region	123

enough to keep up with the pathogen evolution. The downstream signaling response mediated by *R* protein depends on its conformational changes upon perception of an effector or an effector-modified plant protein and the proper oligomerization and interaction with other proteins [124]. There are specific residues in the R gene responsible for the specificity of recognition process [124]. Therefore, by mutating specific residues in the *R* gene, it is possible to manipulate the defense activation output and generate resistance to phylogenetically divergent pathogens, indicating the possibility of engineering durable resistance in crops [125,126] Moreover, transfer of plasma membrane-localized PRR between distinct plant species could confer broad spectrum durable resistance in crops, abolishing the taxonomic restriction [127]. A recent work demonstrated that, expression of Arabidopsis pattern recognition receptor, EFR, in rice enables the rice plant to sense the bacterial ligand of EFR and elicit an immune response [128]. The information gathered for PRRs of diverse origin could possibly be exploited by genome editing tools for developing designer PRRs sensing broad range of pathogens. Thus, modular R receptor having broad spectrum specificities can be developed by leveraging the optimized effector sensing capacity of immune receptors and the versatile programmability of Cas9-gRNA based genome editing [7].

6.2.3. Spatially uncoupling of plant hormones involved in defense response

Plant response against the invading pathogens is under the tight regulation of hormone signalling [129]. There are several plant hormones that are involved in a coordinated immune response. In a general term, salicylic acid (SA) mediated defense response is elicited against the biotrophic and hemi- biotrophic pathogens whereas jasmonic acid (JA) and ethylene (ET) are involved in defense signaling against the necrotrophic pathogens. Both the hormones act antagonistically to each other. Consequently, more often it has been noticed that elevated SA level is correlated with susceptibility to necrotrophic pathogens, and vice versa [129]. Tweaking pathways of hormonal trade-off leading to spatio-temporal uncoupling of antagonistic mode of action of SA and JA/ET might impart broad spectrum resistance against the invading pathogens. This concept was recently explored for the development of black speck resistant tomato variety using CRISPR/Cas9 [130]. In black speck disease, the pathogen *Pseudomonas syringae pv. tomato* (Pto) DC3000 secretes coronatine (COR) that stimulates stomatal opening facilitating the pathogen penetrance and its colonization. In tomato it was found that a co-receptor of COR, SlJAZ2, preferentially accumulates in guard cells and editing of this SLJAZ2 by CRISPR/Cas9 resulted in a dominant repressor protein that prevented stomatal reopening by COR and provided resistance to *PtoDC300*. Moreover, it was interesting to note that in the edited plants the immunity for necrotrophic fungus wasn't compromised. This study provides a method of solving defence trade-off in plants that have several implications in engineering durable and broad-spectrum resistance in crops.

7. CRISPR/CAS: challenges and future prospects

Like most of the biotechnological tools, the techniques of genome editing make use of cellular mechanism but in vitro for the purpose of targeted alteration in the genome. Genome alteration in the process of evolution is beyond our control. However, when the alteration in genome is experimentally done it should be essentially for the beneficial purpose of the society. Its application in crop improvement also should be directed only for those breeding objectives which are absolutely priority and unachievable within the limitation of existing variability. As the case for any new technology, many ethical issues are being raised for genome editing also that need to be addressed by the scientific community. A rational view with adequate support by the regulatory agencies based on scientific principles is essential to harness potential of this technology to the advantage of global agriculture overcoming neophobia of the society.

CRISPR/Cas9 based intentional spread of genetic elements in wild

populations of insect pests that alter the sex ratio of the population or that lead to lethal mutations is a specific and environment friendly strategy to control insect-pests. However, development of insect resistance in response to CRISPR-based gene drive is a real and acute risk at both theoretical [131] and experimental levels [132]. Nevertheless, resistance can be overcome by multiplex genome editing [133]. Thus, it is important to discuss issues related to insect resistance so that scientific and ethical consensus may be reached in favour of this technology.

Further, there are many biosafety concerns related to the release of CRISPR/Cas9-edited insects carrying gene drives in the environment as the modified insects have the potential to bring change in the entire population and/or ecosystem. Therefore, a stringent risk assessment of non-target effects prior to their release is mandatory. The post-release unintended consequences to beneficial insects and/ or pollinators may adversely affect food chains thereby bringing change in the community structure. Furthermore, risk of gene flow between the target species and its non-target counterparts, if any, may worsen the situation. In this regard, a significant reduction in the off-target effects can be achieved through the use of engineered Cas9 variants [21], Cas9 orthologs with distinct DNA binding specificity, two gRNAs in combination with a mutated nickase version of Cas9 [19], dimeric RNA-guided FokI nucleases (RFNs [134]), short guide sequences [135] and guide sequences with no/tolerable non-target activity [136]. The targeted eradication of insect-pests, insect vectors carrying viruses or invasive insect species with gene drive technology may prove to be promising if the associated risks are weighed and addressed appropriately in light of inadvertent ecological consequences. In view of the risk management, use of terminator genes that allow programmed life of edited insects and use of tagged insects for tracking gene flow may prove to be a required step towards utilization of gene drives in the biosafety perspective.

Moreover, use of artificial intelligence and robotic machines to physically kill individual pests [137] is an alternative approach that may be explored for the management of invading pests. However, robots might not be very successful when applied in rough terrains, for hidden eggs and flying or small insects.

CRISPR/Cas9 mediated knock out of susceptibility genes has been successful in imparting disease resistance to invading pathogens. However, one of the serious problems associated with knock out of *S* gene is pleiotropic effects in the plant that often leads to the associated fitness penalties. Nevertheless, editing effector binding element rather than the gene itself provides a viable alternative for achieving disease resistance without affecting the plant's performance [121]. Apart from S gene, stacking of R gene is also a promising method of breeding durable and broad-spectrum resistance in crops. However, transfer and stacking of R gene is extremely laborious and time consuming for tree species because of the protracted generation cycle. For instance, it took more than 50 years to transfer *Malus floribunda* scab resistance gene, *Vf*, to commercial cultivars of apple [138]. The ectopic expression of genes involved in flowering and silencing of key genes regulating flowering may reduce the juvenility period and induce early flowering in tree species [138,139]. Silencing of juvenility inducing genes can be achieved by genome editing and more interestingly once the desirable resistance genes has been transferred the mutation can be segregated in the subsequent generations. Thus, the CRISPR/Cas 9 based approach of engineering disease resistance in tree species will emerge as a powerful tool for delivering genetic traits in commercial cultivars in lesser time. The another way to achieve broad spectrum resistance in crops is to make use of endogenous PRRs and making them capable to recognise diverse pathogenic determinants. In comparison to other approaches this will require a detailed understanding of plant PRRs and their role in eliciting the immune response. In general, it is expected that in future a better understanding of plant immune response and the refinements in genome editing toolbox will allow targeting of more number of immunity players with high specificity which will lead into development of disease resistance cultivars promising a sustainable approach for global agriculture.

8. Concluding remarks

The present era witnesses the advancement of genome editing platforms to a magnitude of bringing revolution in basic and applied plant research. These days CRISPR/Cas technology has gained immense popularity on account of being easy, affordable and versatile genome editing tool. A number of CRISPR-edited plants have the potential to significantly increase crop yields. The dream of creating designer plants with multiple attributes and capable of withstanding all plausible biotic and abiotic challenges are not far away. Thus, this technology should also be explored and optimized for the generation of crop plants immune to insect-pests and pathogens. An initiative and sincere efforts in this regard are vital for saving our crop plants from the enormous yield losses caused due to infestation of insect pests and pathogens. However, fate of the CRISPR-based genome edited products in crop improvement programmes will be majorly decided by regulatory authorities across the world. The regulatory frameworks for new crop varieties follow either process-based or product-based regulation. The extent of regulation imposed on CRISPR-based crops will impact their development costs and also decide the rate at which they enter the commercial markets. The levying of product-based regulations on crops obtained by editing through CRISPR/Cas9 will subject them to be categorized with products obtained through classical mutagenesis consequently clearing them from the restrictions imposed on genetically modified products. This will definitely shape positive public perception for the technology thereby leading to its acceptance by the masses across different nations.

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